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**Hemicalcin, a new toxin from the Iranian scorpion *Hemiscorpius lepturus* which is active on ryanodine-sensitive Ca<sup>2+</sup> channels**

by

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## Abstract

In the present work, we purified and characterized a novel toxin named hemicalcin from the venom of the Iranian chactoid scorpion *Hemiscorpius lepturus* where it represents 0.6% of the total protein content. It is a 33-mer basic peptide reticulated by three disulfide bridges, and that shares between 85 and 91% sequence identity with four other toxins, all known or supposed to be active on ryanodine-sensitive calcium channels. Hemicalcin differs from these other toxins by seven amino acids at positions 9 (L/R), 12 (A/E), 13 (D/N), 14 (K/N), 18 (S/G), 26 (T/A) and 28 (P/I/A). In spite of these differences, hemicalcin remains active on ryanodine-sensitive  $\text{Ca}^{2+}$  channels, since it increases [ $^3\text{H}$ ]-ryanodine binding on RyR1 and triggers  $\text{Ca}^{2+}$  release from sarcoplasmic vesicles. Bilayer lipid membrane experiments, in which the RyR1 channel is reconstituted and its gating properties analyzed, indicate that hemicalcin promotes an increase in the opening probability at intermediate concentration and induces a long-lasting subconductance level of 38% of the original amplitude at higher concentrations. Mice intracerebroventricular inoculation of 300 ng hemicalcin induces neurotoxic symptoms *in vivo*, followed by death. Overall, these data identify a new biologically active toxin that belongs to a family of peptides active on the ryanodine-sensitive channel.

Running title: Hemicalcin, a toxin active on ryanodine receptor

**Key Words:** *Hemiscorpius lepturus*, scorpion toxin, hemicalcin, dihydropyridine receptor, ryanodine receptor, homology modelling, structure-function relationship

## Abbreviations

BLM: bilayer lipid membrane - CNS: central nervous system - DHPR : dihydropyridine receptor - HL: *Hemiscorpius lepturus* - ICK: inhibitor cystine knot – IpTx A: imperatoxin A – JSR: junctional sarcoplasmic reticulum - RyR1: ryanodine receptor type 1 - SR: sarcoplasmic reticulum – TFA, trifluoroacetic acid

## Introduction

*Hemiscorpius Lepturus* (HL) is the most dangerous scorpion of Khuzestan, the south-west, hot and humid province of Iran. Ten to 15% of total scorpion stings during the hot season and almost all cases of scorpion stings during winter are due to *Hemiscorpius lepturus*. These observations are based on a sample of 2534 patients who brought a scorpion specimen to a medical centre while seeking treatment [1]. In addition to inducing typical symptoms of necrosis and ulceration of the skin and haemolysis of blood cells, HL venom exerts its most toxic effects on the central nervous (CNS) and cardiovascular systems [1]. Scorpion peptides that possess a neurotoxic activity in mice are generally known to modulate  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{Ca}^{2+}$  channels. Calcium channels have been classified into various subfamilies including voltage-gated channels, voltage-independent channels and intracellular endoplasmic/sarcoplasmic reticulum calcium release channels. The latter channels include the ryanodine receptors (RyRs) whose openings can be induced by  $\text{Ca}^{2+}$  itself or by an allosteric coupling with the plasmalemmal dihydropyridine receptor (DHPR) [2,3]. Only a few scorpion venom peptides have been identified to possess activity towards ryanodine receptors. *Pandinus imperator* venom contains imperatoxin I and imperatoxin A (IpTx I and A; [4,5]), whereas *Scorpio maurus* contains maurocalcine (MCa; [6]), *Buthus martensii* Karsch contains BmK-PL toxin [7], and *Buthotus judaicus* contains BjTx-1 and BjTx-2 [8]. Although these toxins are potent modulators of RyRs, they are structurally divergent. BjTx-1 and BjTx-2 adopt a classical  $\alpha\beta$  scaffold as many toxins active on voltage-gated  $\text{K}^+$  and  $\text{Na}^+$  channels [9,10], whereas MCa and IpTx A are the only scorpion toxins that adopt an inhibitor cystine knot (ICK) fold [11,12]. In the present work, we isolated, identified and characterized a new neurotoxic peptide from the Iranian scorpion *Hemiscorpius lepturus*. This toxin, named hemicalcin (HCa), differs from MCa and IpTx A by three and four amino acids, respectively. Hemicalcin stimulates [ $^3\text{H}$ ]-ryanodine binding to type 1 RyR (RyR1), produces  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum (SR) vesicles, and triggers significant changes in the biophysical behaviour of RyR1 channels reconstituted in bilayer lipid membranes. Compared to MCa, hemicalcin is significantly more toxic to mice *in vivo*. The data presented herein indicates that hemicalcin represents a new member of the small family of toxins active on RyR1.

## **Experimental**

### **Scorpion venom**

Venoms of *Hemiscorpius lepturus* scorpions from Khuzestan (Iran) were collected by the veterinarian service of the RAZI Vaccine Development and Serum Research Institute of Iran and kept frozen at -20°C in their crude forms until use.

### **Purification of hemicalcin**

Crude venoms were dissolved in water and loaded on Sephadex G-50 gel filtration chromatography columns (2 x K26/50) to isolate a neurotoxic fraction. The columns were equilibrated with 20 mM ammonium acetate at pH 4.7. The neurotoxic fraction was identified by intracerebroventricular (ICV) injection in mice. After lyophilization, the neurotoxic fraction was fractionated by HPLC using a C8 reversed-phase HPLC column (5  $\mu$ m, 4.6 x 250 mm, Beckman Fullerton, CA, USA), equipped with a Beckman Series 125 pump and a Beckman diode array detector set at 214 nm and 280 nm. Elution was controlled by means of the software GOLD. Proteins were eluted from the column at a flow rate of 0.8 ml/min using a linear gradient (45 min) from 12 to 40 % of solution B [0.1 % TFA (trifluoroacetic acid) in acetonitrile] in solution A (0.1 % TFA in water). The protein concentration was measured by the Bradford method.

### **In vivo toxicity tests and LD<sub>50</sub> determination**

The *in vivo* toxicity of hemicalcin was tested on  $20 \pm 2$  g C57/BL6 male mice by intracerebroventricular (ICV) injection of 5  $\mu$ l of 0.1% (w/v) BSA solutions containing increasing amounts of the polypeptides. Six mice were used for each dose; two control mice were injected with only 0.1% BSA in water to be sure that symptoms are not due to experimental conditions. ICV administration was performed under anaesthesia, according to the method described by Galeotti *et al* [13].

### **Amino acid sequence determination of hemicalcin**

Reduction of hemicalcin with dithiothreitol, and alkylation with 4-vinylpyridine, were performed as previously described [14]. The sequence of reduced/carboxymethylated toxin was determined

using an automatic liquid-phase protein sequencer (model 476A, Applied Biosystems) using a standard Edman protein degradation. Hemicalcin was deposited onto Biobrene-precycled glass-fibre discs.

### **Mass spectrometry**

Samples were analyzed on Voyager-DE<sup>TM</sup> PRO MALDI-TOF Workstation mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). The peptide was dissolved in CH<sub>3</sub>CN/H<sub>2</sub>O (30/70) with 0.3% TFA to obtain a concentration of 1-10 pmol/μl. The matrix was prepared as follows: alpha-cyanohydroxycinnamic acid was dissolved in 50% CH<sub>3</sub>CN in 0.3% TFA/H<sub>2</sub>O to obtain a saturated solution of 10 μg/μl. 0.5 μl of peptide solution was mixed with 0.5 μl of matrix solution, placed on the sample plate and the mixture was allowed to dry. Mass spectra were recorded in reflectron mode after external calibration with suitable standards and were analysed using the GRAMS/386 software.

### **Sequence comparison**

Peptides showing sequence similarity with hemicalcin were identified with BLAST2 [15] in the non redundant database. Best hits were calcium channel toxin modulators. The amino acid sequences of these peptides were aligned and identities determined.

### **SR vesicles preparation**

Heavy SR vesicles were prepared following a modified method of Kim and collaborators [16] as described in Marty *et al.* [17]. Junctional SR was prepared as described by Pessah *et al.* [18]. Protein concentration was measured by the methods of Biuret or Lowry.

### **[<sup>3</sup>H]-ryanodine binding assay**

Heavy SR vesicles (1 mg/ml) were incubated at 37°C for 3 hrs in an assay buffer composed of 5 nM [<sup>3</sup>H]-ryanodine, 150 mM NaCl, 2 mM EGTA, 2 mM CaCl<sub>2</sub> (pCa=5), and 20 mM HEPES, pH 7.4. Hemicalcin was added to the assay buffer just prior the addition of heavy SR vesicles. [<sup>3</sup>H]-ryanodine bound to heavy SR vesicles was measured by filtration through Whatmann GF/B glass filters followed by three washes with 5 ml of ice-cold washing buffer composed of 150 mM NaCl, 20 mM HEPES, pH 7.4. Filters were then soaked overnight in 10 ml scintillation cocktail

(Cybscint, ICN) and bound radioactivity determined by scintillation spectrometry. Non-specific binding was measured in the presence of 20  $\mu$ M unlabelled ryanodine. Each experiment was performed in triplicate and repeated at least two times. All data are presented as mean  $\pm$  S.D.

### **Ca<sup>2+</sup> release measurements**

Ca<sup>2+</sup> release from heavy SR vesicles was measured using antipyrylazo III, a Ca<sup>2+</sup>-sensitive dye. The absorbance was monitored at 710 nm by a diode array spectrophotometer (MOS-200 Optical System, Biologic, Claix, France). 25  $\mu$ g of heavy SR vesicles were actively loaded with Ca<sup>2+</sup> at 37°C in a 2 ml buffer containing: 100 mM KCl, 7.5 mM sodium pyrophosphate, 20 mM potassium 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0, supplemented with 250  $\mu$ M antipyrylazo III and 1 mM ATP/MgCl<sub>2</sub>. Ca<sup>2+</sup> loading was started by four sequential additions of 20  $\mu$ M CaCl<sub>2</sub>. In these loading conditions, there was no calcium-induced calcium release to interfere with the effect of hemicalcin on Ca<sup>2+</sup> release from heavy SR vesicles. At the end of the experiment, the Ca<sup>2+</sup> released by hemicalcin was chelated by the addition of 1 mM EGTA.

### **Recording and analysis of RyR1 single channel activity in bilayer lipid membrane (BLM)**

The BLM was formed across a 200  $\mu$ m aperture drilled into a polysulfone cup (Warner Instrument Corp., CT) using a mixture composed of phosphatidylethanolamine: phosphatidylserine: phosphatidylcholine (5:3:2 w/w, Avanti Polar Lipids, Inc, AL) dissolved in decane at a final concentration of 30 mg/ml. The BLM partitioned two chambers (*cis* and *trans*) with buffer solution (in mM): 500 CsCl, 7  $\mu$ M free Ca<sup>2+</sup>, and 20 HEPES-Tris (pH 7.4) on *cis*, 50 CsCl and 20 HEPES-Tris (pH 7.4) on *trans*. The *cis* chamber was virtually grounded, whereas the *trans* chamber was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instrument, CT). Single channels were obtained by introducing rabbit skeletal muscle junctional SR (JSR) vesicles or purified RyR1 preparation in the *cis* chamber to induce fusion with the BLM. Immediately after incorporation of a channel into the BLM, the *cis* chamber was perfused with an identical solution to prevent additional vesicle fusions. Single channel gating was monitored and recorded at a holding potential of -40 mV (applied to the *trans* side). The amplified current signals, filtered at 1 kHz (8 Pole Low-Pass Bessel Filter, Warner Instrument, CT) was digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). All the recordings were made for at least 3 min under each

defined condition. The channel open probability ( $P_o$ ), mean open-, mean closed-dwell times ( $\tau_o$  and  $\tau_c$ ) and current amplitude histograms were calculated and graphically displayed using Clampfit, pClamp software 9.0 (Axon-Molecular Devices, Union City, CA).

### **Molecular modelling**

A structural model of hemicalcin was generated by homology modelling with the program Modeller 8v2 [19]. Homologous polypeptides with known structures were identified by a Blast2 [15] search of the PDB database (RCSB organization) using the sequence of hemicalcin as entry. The solution structure of MCa (PDB code 1C6W) [11] and IpTx A (PDB code 1IE6) [12] were used as templates. Twenty structures were calculated, from which only one model, combining the best Ramachandran plot (<http://swift.cmbi.kun.nl/WIWWI/ramaplot.html>; [20]), and good scores for the objective function values [19], and the VICTOR/FRST energy function proposed by Tossato (2005) (<http://protein.cribi.unipd.it/frst/>; [21]), was selected. The model was then visualized with the ViewerLite50 program (<http://www.accelrys.com/products/dstudio/>).



## RESULTS

### Purification of hemicalcin

After Sephadex G50-chromatography of the *Hemiscorpius lepturus* venom, each fraction obtained was injected to the mice by ICV route and the neurotoxic fractions were pooled (Figure 1A) and applied to a C8 HPLC column. Hemicalcin was eluted at 18.93 min (Figure 1B). An analytical HPLC run of hemicalcin showed a single symmetric peak. Hemicalcin represents about 0.6% of the *Hemiscorpius lepturus* venom.

### *In vivo* toxicity of hemicalcin

Hemicalcin (500 ng in 5  $\mu$ l) was injected into mice using the ICV route and the following symptoms were observed. The toxin produced rapid paralysis in the lower half of the body. The mice are no longer able to move their head forward. The mice turn backward while moving circularly, and their head are down to the ground and bent to the body. Five to ten minutes after injection, the first symptoms disappear and the mice start to jump and run very fast. This phenomenon takes 1 to 2 min. The animals then stop moving and start trembling for a while. Next, they take a rest without any movement. Jumping, running and trembling happens every 5 to 10 min. The last stage is repeated 4 to 5 times and in the last trembling episode, spasms occur in all muscles and the animals die while their bodies are laid down and their four legs are in a straight position. With sublethal doses of hemicalcin, the same symptoms were observed except the very last stage that precedes death. The 50% killing dose for mice is of 300 ng, giving an LD50 of 15  $\mu$ g toxin/ 1 Kg mouse.

### Sequence determination and comparison to other scorpion toxins

Edman degradation of 2 nmoles of S-pyridyl-ethylated peptides led to the identification of the complete amino acid sequence of the peptide (Figure 2). Hemicalcin is a peptide composed of 33 amino acid residues containing six cysteine residues. The experimental molecular mass of native hemicalcin ( $3788.09 \pm 0.67$  Da) obtained by MALDI is nearly identical to the average theoretical molecular mass calculated for the fully oxidized form of hemicalcin (3787 Da). These results indicate that the six cysteines of hemicalcin are engaged in three intramolecular disulfide bridges.

Moreover, only a monomeric form was observed in the mass spectra, suggesting the absence of intermolecular disulfide bridges.

Hemicalcin has very strong sequence homologies with MCa from the chactoid scorpion *Scorpio maurus* [6] (91% sequence identity), with IpTx A from the scorpion *Pandinus imperator* [5] (88% sequence identity), and with both opicalcine 1 and 2 from the scorpion *Opisthophthalmus carinatus* [22] (88% and 85% sequence identity, respectively) (Figure 2). Among these toxins, variation in amino acid sequence takes place at seven positions (9, 12-14, 18, 26, and 28) without altering the net global charge of the peptides (+8). The alignment of the cysteine residues of hemicalcin with that of MCa and IpTx A, indicates that hemicalcin could also possess the typical pairing of disulfide bridges observed in the family of toxins belonging to the family *scorpionidae* (Cys3-Cys17, Cys10-Cys21, and Cys16-Cys32).

Like the four other scorpion toxins, hemicalcin also presents a sequence homology (21 % identity) with peptide A which is derived from the cytoplasmic II-III loop of the  $Ca_v1.1$  subunit of the DHPR (Figure 2B).

### **[ $^3H$ ]-ryanodine binding assay**

MCa and IpTx A are both known to stimulate the binding of [ $^3H$ ]-ryanodine, a biochemical indicator of channel activity, to heavy SR vesicles enriched in RyR1 [5,11]. Considering the sequence homology among these toxins, it is expected that hemicalcin should also enhance [ $^3H$ ]-ryanodine binding onto RyR1. Figure 3 demonstrates that hemicalcin does indeed increase the binding of [ $^3H$ ]-ryanodine in a dose-dependent manner with an  $EC_{50}$  of  $71 \pm 6$  nM. Under these assay conditions, the binding of [ $^3H$ ]-ryanodine on RyR1 was stimulated by 11.8 fold at saturating concentrations of hemicalcin. By comparison, MCa was slightly more effective than hemicalcin since it increased [ $^3H$ ]-ryanodine binding by 16.7-fold with an  $EC_{50}$  of  $25 \pm 2$  nM.

### **Enhancement of $Ca^{2+}$ release from SR**

In order to investigate the effect of hemicalcin on  $Ca^{2+}$  release from heavy SR vesicles, we first determined that the purified hemicalcin did not contain any significant  $Ca^{2+}$  concentration that could affect the interpretation of  $Ca^{2+}$  release measurements induced by the peptide. Absorbance measurements indeed illustrate the absence of significant  $Ca^{2+}$  concentration upon addition of 40 nM hemicalcin (Fig. 4A). Next, we actively loaded the SR vesicles by four consecutive additions

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of  $\text{Ca}^{2+}$  (20  $\mu\text{M}$ ) in the presence of ATP- $\text{Mg}^{2+}$ , pyrophosphate and ATP regenerating system. After  $\text{Ca}^{2+}$  loading reaches equilibrium, addition of 125 nM hemicalcin along with 20  $\mu\text{M}$   $\text{Ca}^{2+}$  to the external medium induces  $\text{Ca}^{2+}$  release as shown in Figure 4B. The hemicalcin response comes with a slower up rise kinetics than the added  $\text{Ca}^{2+}$  again indicating that could not be linked to contaminating  $\text{Ca}^{2+}$  from the purified material. External  $\text{Ca}^{2+}$  level reaches a plateau level indicating that near maximal  $\text{Ca}^{2+}$  release had occurred.  $\text{Ca}^{2+}$  was added along hemicalcin as it appears to promote the effect of the peptide. The remaining  $\text{Ca}^{2+}$  from the SR vesicles could be released by the application of 1  $\mu\text{M}$  A23187 ionophore. Further application of 20  $\mu\text{M}$  external  $\text{Ca}^{2+}$  were not taken up by the vesicles, coherent with the effect of the ionophore in SR vesicles, which is to produce a continuous  $\text{Ca}^{2+}$  leakage from the vesicles. The external  $\text{Ca}^{2+}$  concentration can be lowered by the application of 0.5 mM external EGTA. The  $\text{Ca}^{2+}$  specifically released by hemicalcin from the SR vesicles amounted to  $47.7 \pm 15.1$  nmol ( $n=3$ ) and represented  $66 \pm 18\%$  of the total calcium stored by the vesicles. As control experiments, and in order to make sure that the fifth application of 20  $\mu\text{M}$   $\text{Ca}^{2+}$  is not responsible for the large response of hemicalcin, an experiment was performed in which five consecutive application of 20  $\mu\text{M}$   $\text{Ca}^{2+}$  were applied to SR vesicles (Figure 4C). As indicated, 20  $\mu\text{M}$   $\text{Ca}^{2+}$  alone for the fifth application does not produce a response similar than 20  $\mu\text{M}$   $\text{Ca}^{2+}$  along with hemicalcin. This was also not the case when the fifth application was 40  $\mu\text{M}$   $\text{Ca}^{2+}$ , totally precluding the idea that hemicalcin is contaminated with calcium. Overall, these results are similar to those previously observed with Mca and imperatoxin A indicating a possible direct effect of hemicalcin on RyR1 [23, 24]. To further illustrate this point, the effect of hemicalcin on RyR1 channel activity was investigated.

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### Effects of hemicalcin on RyR1 channel activity

The mechanism by which hemicalcin influences RyR1 channel activity was further assessed by measuring the effects of the toxin on the gating parameters of single channels reconstituted into BLM. Figure 5 shows a RyR1 channel in the presence of 7  $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  before (Panel A) and after (Panels B & C) being exposed to nanomolar concentrations of hemicalcin. In the absence of hemicalcin, the channel exhibited low activity with an open probability ( $P_o$ ) of 0.039 (Panel A). However, addition of 175 nM hemicalcin into *cis* solution rapidly activated the channel, promoting a 2.1-fold increase of the  $P_o$  to 0.083 and moderate changes in both the mean open-, closed-dwell time values ( $\tau_o$  and  $\tau_c$ ):  $\tau_o$  increased by 124% whereas  $\tau_c$  decreased by 59%.

A prominent effect of hemicalcin (175 nM) on channel gating behaviour was stabilization of an occasional long-lasting sub-conductance state. Amplitude histogram analysis indicates that the predominant hemicalcin-modified sub-conductance state was ~38 % of the full state of 37.2 pA. The hemicalcin-modified channel displayed a mean open time of  $\tau_o = 0.92 \pm 0.32$  sec, more than 1600-fold longer than that of the native RyR1 channel gating mode. The occurrence ( $F_o$ ) of this sub-conductance gating is ~2.7 % (the percentage of the time that the channel gates in a sub-conductance state *versus* total time, all-mode gating states). A higher concentration of hemicalcin (350 nM final concentration in *cis* solution, Panel C) did not further significantly change the  $P_o$ ,  $\tau_o$  and  $\tau_c$ , but resulted in more frequent appearance of the long-lasting sub-conductance gating mode:  $F_o$  increased more than 4-fold from 2.7 % to 11.1 %. The subsequent addition of ryanodine (125  $\mu$ M) into the *cis* solution of the hemicalcin-modified channel, rapidly stabilized a sub-state approximately 60 % of the full conductance state (ryanodine-modified substate), with frequent closures to a current level 20.9 % of the maximum current level (ryanodine + hemicalcin-modified state). Under these conditions, the channel shut down completely within 45 sec (data not shown).

In other BLM experiments, 50 nM hemicalcin was found to produce negligible effect on RyR1 channel gating kinetics ( $n=3$ ); whereas at 100 nM the toxin slightly increased  $P_o$  and induced the occasional appearance of long-lasting sub-conductance gating mode indicating that the effects of hemicalcin on channel gating are dose-dependent (data not shown).

### **Molecular model of hemicalcin**

The molecular model of hemicalcin was established by homology modelling using solution structures of MCa and IpTx A as templates. The molecular model of hemicalcin is shown along the 3-D solution structure of MCa (Figure 6A). As expected, the fold of the HCa model appears to be very similar to the folding of the template experimental structure : i.e. a compact disulfide-bonded core from which several loops and the N-terminus emerge. The main elements of regular secondary structure are a double-stranded antiparallel  $\beta$ -sheet comprising residues 20-22 and 30-33, a third peripheral extended strand composed of residues 8-10, which is almost perpendicular to the double-stranded antiparallel  $\beta$ -sheet, and a helical turn composed of residues 13-15. Thus, the model suggests that hemicalcin probably folds according to the inhibitor cystine knot (ICK) previously described in MCa [11] and IpTx A [12].



## Discussion

In this study, we isolated the first toxin from the venom of the Iranian scorpion *Hemiscorpus lepturus*, herein termed hemicalcin. It is a highly basic 33 amino acid residue peptide that is cross-linked by three disulfide bridges. It shares about 90% sequence identity with MCa and 88% with IpTx A. Like these two related toxins, hemicalcin also shares sequence homology with peptide A, a domain of the II-III loop of  $\text{Ca}_v1.1$ , a dihydropyridine-sensitive voltage-dependent calcium channel of the skeletal muscle. A common binding site on RyR1 that can bind peptide A as well as MCa indicates that the binding of these toxins on RyR1 involves the region of homology between peptide A and the toxins [25]. Peptide A induces  $\text{Ca}^{2+}$  release from SR and enhances current flow through RyR1 channels [5,26-28]. The ability of peptide A to activate RyR1 is highly related on the orientation of its positively charged residues along one face of the molecule [28]. Both IpTx A and MCa also share a similar orientation of positively charged residues with peptide A in this surface [11,28], indicating that a same basic domain at the surface of these peptides is most likely involved in the activation of the RyR1 channel. The molecular model of hemicalcin suggests that it also possesses a highly basic domain that presents the same surface orientation as in IpTx A and MCa. Because of its high homology with peptides active on RyR1 channels, we expected that hemicalcin could be able to activate this channel type too. Indeed, hemicalcin was found to increase [ $^3\text{H}$ ]-ryanodine binding on RyR1 with an apparent  $\text{EC}_{50}$  of  $71 \pm 6$  nM. In comparison, hemicalcin was found almost as potent as MCa for stimulating [ $^3\text{H}$ ]-ryanodine binding. Also, application of 50 nM hemicalcin on SR-loaded with  $\text{Ca}^{2+}$  produced a significant  $\text{Ca}^{2+}$  release. Enhanced release of  $\text{Ca}^{2+}$  and specific [ $^3\text{H}$ ]-ryanodine binding is due to a direct effect of hemicalcin on the gating properties of RyR1 channel since the peptide induces an increase in opening probability and, at higher concentrations, stabilizes a long-lived sub-conductance state. These results show that the activity of hemicalcin on RyR1  $\text{Ca}^{2+}$  channels is similar to those observed with the two other toxins characterized hitherto, indicating that these toxins have similar mechanisms of action on RyR1. However, the predominant sub-conductance level that hemicalcin induces (38% of full conductance) differs significantly from those measured with MCa (~48-54% of full conductance; [23,24]) or IpTx A (25% of full conductance; [29,30]). The functional differences observed among these toxins must be endowed by their structural differences.

Structure-function relationships of calcium channel scorpion toxins, especially on RyR isoforms specificity, are not yet well defined because only few members of these toxins were found within a scorpion specie. Until now only two toxins MCa and IptxA (from *scorpio maurus* and *Pandinus Imperator* scorpions, respectively) derived from orthologous genes, were studied. The discovery of new natural toxins from related species might give access to active structures displaying multipoint mutations compared with these two toxins and may bring useful information in structure-function relationships. Previous study on synthetic monosubstituted analogues of MCa, showed that some amino acid residues of MCa can be classified in three different groups : (i) residues belonging to the basic class that are important for interaction with RyR1 (Lys<sub>22</sub>, Arg<sub>23</sub> and Arg<sub>24</sub>); (ii) residues belonging or not to the basic class that appear less important for the interaction with RyR1 (Lys<sub>19</sub>, Lys<sub>20</sub> and Thr<sub>26</sub>); and (iii) a residue of the acidic face that appears to mildly affect the interaction with RyR1 (Lys<sub>8</sub>) [23]. All these residues are conserved in hemicalcin; only 3 amino acids are different between hemicalcin and MCa (A<sub>12</sub>/E<sub>12</sub>, D<sub>13</sub>/N<sub>13</sub> and P<sub>28</sub>/I<sub>28</sub>), whereas 4 differences are noted between hemicalcin and imperatoxin A (L<sub>9</sub>/R<sub>9</sub>, K<sub>14</sub>/N<sub>14</sub>, S<sub>18</sub>/G<sub>18</sub> and P<sub>28</sub>/A<sub>28</sub>). Compared to the MCa experimental structure, the molecular model of hemicalcin suggests that the A<sub>12</sub>/E<sub>12</sub>, D<sub>13</sub>/N<sub>13</sub> and P<sub>28</sub>/I<sub>28</sub> mutations have no significant effect on the backbone structure. Careful examination of the side-chains of these residues showed that D<sub>13</sub> likely interacts with five Lys residues (K<sub>14</sub>, K<sub>19</sub>, K<sub>20</sub>, K<sub>22</sub>, and K<sub>30</sub>) in HCa. The interaction between D<sub>13</sub> and K<sub>30</sub> may be especially privileged, thus allowing K<sub>14</sub> to be well exposed. In IpTx A, D<sub>13</sub> interacts with only four Lys residues (K<sub>19</sub>, K<sub>20</sub>, K<sub>22</sub>, and K<sub>30</sub>), whereas in MCa, E<sub>12</sub>, which does not possess a homologous, but rather an adjacent relative (D<sub>13</sub>) in HCa, interacts also with four Lys residues (K<sub>14</sub>, K<sub>11</sub>, K<sub>19</sub>, and K<sub>30</sub>). Although the net global charge of MCa and Iptx A is conserved in HCa, a double mutation in the 9-14 region (D<sub>13</sub>/N<sub>13</sub> and A<sub>12</sub>/E<sub>12</sub>, L<sub>9</sub>/R<sub>9</sub> and K<sub>14</sub>/N<sub>14</sub>) may change its electrostatic environment. This property may explain the fact that hemicalcin exhibits a lower potency on RyR1 compared to MCa and IpTx A.

The differences in toxicity of hemicalcin and of the two related toxins are also interesting to note. According to their respective LD<sub>50</sub>, hemicalcin (LD<sub>50</sub> = 15 µg toxin/1 Kg mouse) is 67 times more toxic on mice than MCa (LD<sub>50</sub> = 400 µg toxin/1 Kg mouse [6]) by intracerebroventricular inoculation. The neurotoxic symptoms observed in mice suggest that hemicalcin is active on the CNS, but this requires further investigations. Among different hypothesis that may explain these differences, one suggests that hemicalcin is more resistant to proteolysis, more efficient in terms

of cell penetration to reach its pharmacological target, or possibly more potent on related RyR isoforms, such as RyR3 or RyR2 that are predominantly expressed in the CNS.

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## Figures

### Figure 1. Purification of hemicalcin from the venom of *Hemiscorpius lepturus*

A) The extracted venom was fractionated by G-50 gel filtration chromatography columns (2 x K26/50) equilibrated with 20 mM ammonium acetate pH 4.7.

B) Hemicalcin was purified from the neurotoxic fraction (II') by RP-HPLC on a C8 column using a gradient of buffer B (0.1% TFA in acetonitrile) as described in the Experimental section. Hemicalcin was collected at 18.93 min.

### Figure 2. Sequence alignment of hemicalcin with related toxins active on the ryanodine receptor

A) Sequence alignment of hemicalcin with four analogous toxins, MCa, IpTx A, opicalcine 1 and opicalcine 2. MCa and IpTx A are two peptides known to be active on the ryanodine receptor. Opicalcine 1 and 2 have not been tested for pharmacological activity. All five toxins have the same number of positively charged amino acid residues (the N-terminal glycine residue, six or seven lysine residues, and five or four arginine residues; indicated in blue). Negatively charged amino acids are represented in red.

B) A sequence alignment of hemicalcin with peptide A of the II-III loop of  $\text{Ca}_v\alpha_{1.1}$  subunit from the dihydropyridine receptor (DHPR) is also provided. The strongest homology stretches from Lys<sup>19</sup> to Thr<sup>26</sup> of hemicalcin.

### Figure 3. Hemicalcin affects [<sup>3</sup>H]-ryanodine binding on heavy SR vesicles

Dose-dependent effect of hemicalcin on [<sup>3</sup>H]-ryanodine binding onto heavy SR vesicles (open circles). [<sup>3</sup>H]-ryanodine binding was measured at pCa 5 in the presence of 5 nM [<sup>3</sup>H]-ryanodine for 3 hrs at 37°C. Non-specific binding remained constant at all hemicalcin concentrations and was less than 200 fmol/mg. Data were fitted with a logistic function  $y = y_0 + (a / (1 + (x/EC_{50})^b))$  where  $y_0 = 316 \pm 46$  in the absence of hemicalcin,  $a = 2799 \pm 118$  fmol/mg is the maximum [<sup>3</sup>H]-ryanodine binding,  $b = -1.1 \pm 0.1$  the slope coefficient, and  $EC_{50} = 71 \pm 6$  nM the concentration of hemicalcin for half stimulation of [<sup>3</sup>H]-ryanodine binding. Overall, hemicalcin stimulates [<sup>3</sup>H]-ryanodine binding by a factor of 11.8-fold at saturation level. For comparison, the data are also

shown for MCa (filled circles;  $y_0 = 364 \pm 129$ ,  $a = 3502 \pm 127$  fmol/mg,  $b = -1.0 \pm 0.1$ , and  $EC_{50} = 25 \pm 2$  nM. Stimulation factor of 16.7 at saturation).

#### Figure 4. $Ca^{2+}$ release from heavy SR vesicles induced by hemicalcin

A) Absorbance measured in the absence of SR vesicles and in response to sequential  $Ca^{2+}$  additions in the medium or to 40 nM hemicalcin addition. The data indicate the lack of  $Ca^{2+}$  presence in the purified material.

B) Heavy SR vesicles were actively loaded with  $Ca^{2+}$  by four sequential additions of 20  $\mu$ M  $CaCl_2$  in the monitoring chamber. The absorbance was monitored to show that the added  $Ca^{2+}$  was taken up by the SR vesicles. The trace relaxed close to its original baseline with  $CaCl_2$  additions constituting approximately 70-80% of the SR loading capacity. These  $Ca^{2+}$  additions were used to calibrate the  $Ca^{2+}$  release. Addition of 125 nM hemicalcin produces a long lasting  $Ca^{2+}$  release from SR vesicles. 20  $\mu$ M  $Ca^{2+}$  was added along hemicalcin to control the extravesicular concentration of  $Ca^{2+}$ . Also, external  $Ca^{2+}$  appears to act as a cofactor to hemicalcin effect. Residual  $Ca^{2+}$  from SR vesicles is released by the addition of 1  $\mu$ M of the ionophore A23187. Further addition of 0.5 mM EGTA buffers the released and the basal  $Ca^{2+}$  from the system.

C) Loading of heavy SR vesicles with  $Ca^{2+}$  by five sequential additions of 20  $\mu$ M  $CaCl_2$  in the monitoring absorbance chamber. The fifth application does not produce a similar calcium release as the one observed by the co-application of 20  $\mu$ M  $Ca^{2+}$  and 125 nM hemicalcin in B).

D) Loading of heavy SR vesicles with  $Ca^{2+}$  by four sequential additions of 20  $\mu$ M  $CaCl_2$  and a fifth one at 40  $\mu$ M in the monitoring absorbance chamber. The fifth application produces a slower loading of the vesicles but no sustained response.

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#### Figure 5. Hemicalcin alters gating kinetics and stabilizes subconductances of RyR1 single channel activity in bilayer lipid membrane

RyR1 single channel was incorporated by inducing fusion of skeletal muscle junctional SR vesicles into BLM. The channel activities were recorded and analyzed as described in the *Materials and Methods*. RyR1 single channel in the absence of hemicalcin was used to serve as control and was recorded for 3 min (A). Sequential additions of hemicalcin to achieve a final concentration of 175 nM (B) and 350 nM (C) were made into *cis* chamber, and the channel

activity was recorded for 5 min under each condition. The dashed lines indicate the maximum current amplitude of the native RyR1 channel (37.2 pA) when the channel is fully open (o). The arrow indicating “c” shows the zero current level when the channel is in the fully closed state, and the arrow indicating “s” shows the hemicalcin-stabilized sub-conductance state of the channel. The terms of  $P_o$ ,  $\tau_o$ ,  $\tau_c$  and  $F_o$  represent open probability, mean open-dwell time, mean closed-dwell time and occurrence of stable sub-conductance in the total recorded period, respectively. The data is representative of a total of five independent bilayer experiments with RyR1 channels from one junctional SR protein preparation and one purified RyR1 preparation.

### **Figure 6. Homology model of hemicalcin**

Backbone ribbon representation of the model of hemicalcin and comparison with the structure of MCa (PDB code 1C6W [11]), which, besides IpTx A, is one of the two structures used as template for molecular modelling. Disulfide bridges are in stick representation. Basic amino acid residues present on the same face of MCa and hemicalcin ( $K^{19}$ ,  $K^{20}$ ,  $K^{22}$ ,  $R^{23}$ ,  $R^{24}$  and  $K^{30}$ ) are shown. The side-chain bonds of  $K^{11}/K^{14}/D^{13}/A^{12}$  in hemicalcin and  $K^{11}/K^{14}/N^{13}/E^{12}$  in MCa are also shown.